Endonuclease heteroduplex mismatch cleavage for detecting mutation genetic variation of trypsin inhibitors in soybean

Gordana Petrović[(1)], Zorica Nikolić[(1)], Vuk Đorđević[(1)], Vesna Župunski[(1)], Dušica Jovičić[(1)], Maja Ignjatov[(1)] and Dragana Milošević[(1)]

[(1)]Institute of Field and Vegetable Crops, Maksima Gorkog 30, Novi Sad, Serbia. E-mail: gordana.zdjelar@nsseme.com, zorica.nikolic@nsseme.com, vuk.djordjevic@nsseme.com, dusicajovicic@hotmail.com, maja.ignjatov@nsseme.com, dragana.petrovic@nsseme.com

Abstract – The objective of this work was to evaluate the genetic variation of trypsin inhibitor in cultivated (*Glycine max* L.) and wild (*Glycine soja* Siebold & Zucc.) soybean varieties. Genetic variations of the Kunitz trypsin inhibitor, represented by a 21-kD protein (KTI), and of the Bowman-Birk trypsin–chymotrypsin inhibitor (BBI) were evaluated in cultivated (*G. max*) and wild (*G. soja*) soybean varieties. Endonuclease heteroduplex mismatch cleavage assays were performed to detect mutations in the KTI gene, with a single-stranded specific nuclease obtained from celery extracts (CEL I). The investigated soybean varieties showed low level of genetic variation in KTI and BBI. PCR-RFLP analysis divided the BBI-A type into subtypes A1 and A2, and showed that Tib type of KTI is the dominant type. Digestion with restriction enzymes was not able to detect differences between ti-null and other types of Ti alleles, while the endonuclease heteroduplex mismatch cleavage assay with CEL I could detect ti-null type. The digestion method with CEL I provides a simple and useful genetic tool for SNP analysis. The presented method can be used as a tool for fast and useful screening of desired genotypes in future breeding programs of soybean.

Index terms: *Glycine max*, *Glycine soja*, antinutritional factors, protease inhibitors, SNP.

Endonuclease com incompatibilidade heteroduplex para detectar mutação e variações genéticas de inibidores da tripsina em soja

Resumo – O objetivo deste trabalho foi avaliar a variação genética do inibidor de tripsina em variedades cultivadas (*Glycine max*) e silvestres (*Glycine soja*) de soja. Foram avaliadas as variações genéticas do inibidor de tripsina Kunitz, representado pela proteína 21-kDa (KTI), e do inibidor de tripsina-quimotripsina Bowman-Birk (BBI), em variedades de soja cultivadas (*G. max*) e selvagens (*G. soja*). Ensaios de clivagem foram feitos com endonuclease de incompatibilidade heteroduplex, para a detectar mutações no gene de KTI, com uma única nuclease específica de cadeia simples, obtida a partir de extractos de aipo (CEL I). As variedades de soja estudadas apresentaram baixo nível de variação genética em KTI e BBI. A análise por PCR -RFLP dividiu o BBI-A em A1 e A2 e mostrou que o Tib do KTI é o tipo dominante. A digestão com enzimas de restrição não foi capaz de detectar diferenças entre os tipos de ti-null e outros alelos Ti, enquanto o ensaio com endonucleases com incompatibilidade heteroduplex com CEL I pôde detectar o tipo ti-null. O método de digestão com CEL I fornece uma ferramenta genética simples e útil para a análise de SNP. O método apresentado pode ser utilizado como ferramenta para a triagem rápida e útil de genótipos desejáveis em futuros programas de melhoramento de soja.

Termos para indexação: *Glycine max*, *Glycine soja*, factores antinutritionais, inibidores de protease, SNP.

Introduction

In general, most commercial soybean cultivars contain about 40% protein and represent an important source of this nutrient (Song et al., 2013). Soybean proteins have traditionally been used for animal feed; however, their use for human consumption are increasing (Livingstone et al., 2007). Nevertheless, raw soybean cannot be used for animal feeding because of the presence of some antinutritional factors that decrease its nutritional value. Protease inhibitors, which represent about 6% of the total seed protein content, are among the main antinutritional factors in soybean seeds (Wang et al., 2004). There are two major classes of protease inhibitors: the Kunitz trypsin inhibitor (KTI), represented by a 21-kD protein, and the Bowman-Birk trypsin–chymotrypsin inhibitor (BBI), which consists of several related 8-kD proteins

(Kim et al., 2010). Approximately 80% of the trypsin inhibition activity is caused by KTI (Barros et al., 2008).

The biological roles of protease inhibitors are not clear. A number of functions has been proposed for BBIs, including the regulation of protease activity during seed germination and the protection of plants from insects and microorganisms. Moreover, BBIs may also function as storage of sulfur amino acids (Barros et al., 2012; Cruz et al., 2013). Recent investigations have focused on its medicinal utility for suppressing both initiation and promotion stages of carcinogenesis (Rakashanda & Amin, 2013). BBI protease inhibitors are double-headed serine protease inhibitors that bind both enzymes at two independent reactive sites with a network of highly conserved disulfide bridges (Barros et al., 2012).

Genes encoding BBI in *Glycine max* and *Glycine soja* are a multigene family, with at least five members: BBI-A, BBI-B, BBI-CII, BBI-DII and BBI-EI. BBI-B is supposed to be encoded by a gene which is closely related to BBI-A, designated as BBI-A2, and both are very similar. Post-translational proteolysis indicates that BBI-EI is originated from BBI-DII. Thus, BBIs are grouped in three types with distinct characteristics: BBI-A, BBI-C, and BBI-D (Deshimaru et al., 2004; Wang et al., 2008; Barros et al., 2012).

Based on electrophoretograms, 12 forms of KTI have been found: *Tia* and *Tib* (Wang et al., 2008, 2010), *Tic* (Hymowitz, 1973), *Tid* (Zhao & Wang, 1992), *Tie* (Wang et al., 2008), *Tif* (Wang et al., 2004), Tib^{i5} (Wang et al., 2008), Tia^{a1}, Tia^{a2}, Tia^{b1}, *Tig* (Wang et al., 2008) and *ti*-null type (Orf & Hymowitz,1979). Of these, *Tia* and *Tib*, which differ by nine amino acids, are the predominant types (Lee et al., 2012). These several polymorphic soybean KTI types are controlled by codominant multiple alleles at a single locus (Wang et al., 2008). There are at least ten distinct DNA sequences in soybean genome for the Kunitz trypsin inhibitor, some of which occur in tandem pairs (Jofuku & Goldberg, 1989). At least three of these have been confirmed to represent functional genes, referred to as KTI1, KTI2 and KTI3. The major Kunitz trypsin inhibitor gene in soybean seeds is KTI3 (Jofuku & Goldberg 1989). The null phenotype of KTI, which has reduced amounts of Kunitz trypsin inhibitor and lacks detectable Kunitz trypsin inhibitor activity, is inherited as a recessive allele *ti* (Orf & Hymowitz, 1979). This null line has three mutations: two deletions and one G → T transversion occurred within the KTI3 gene (Jofuku et al., 1989). These mutations cause a translational frameshift that results in four stop codons to be inserted into the KTI3 mRNA reading frame and premature termination of KTI3 mRNA translation, and leads to a 100-fold reduction of KTI3 mRNA in soybean embryos (Jofuku et al., 1989).

There are some correlations between a single nucleotide polymorphism (SNP) and many of the key characters of crops. Even the phenotype of some characters can be indicated by SNPs. Therefore, detecting SNPs for important functional genes and identifying their relationship with desired phenotypes are important tools in plant breeding program. According to the demand, it may be necessary to develop soybean cultivars with a high content of inhibitors for resistance to insects, or to develop cultivars with a reduced content of inhibitors for a better nutritive value. One of the methods for SNP detection is a heteroduplex mismatch cleavage assay using the endonuclease CEL I from celery in plants (Zolala et al., 2009), animals (Kuroyanagi et al., 2013), and humans (Till et al., 2006). CEL I is a mannosyl glycoprotein which cuts the 3' side of the loops formed in double-stranded heteroduplex DNA molecules, at sites of base substitutions, and small insertion or deletions (indels) (Yang et al., 2004). CEL I endonuclease assay has proved to be useful for SNP detection in tomato (Yang et al., 2004), wheat (Chen et al., 2011), common bean (Galeano et al., 2009), and sunflower (Fusari et al., 2011).

The objective of this work was to evaluate the genetic variation of trypsin inhibitor in cultivated (*Glycine max*), and wild (*Glycine soja*) soybean varieties.

Materials and Methods

Ten soybean varieties were selected, five of them from USDA (Altona, Wilkin, Amsoy 71, Panther, and Kunitz); two from the Institute of Field and Vegetable Crops, Soybean Breeding Program from Novi Sad, Serbia ('Vojvodjanka' and 'Fortuna'); and three were wild soybean varieties (*G. Soja*) provided by N.I. Vavilov Research Institute collection, from St. Petersburg, Russia (37-2, Primorye district, Russia; 42-2, Khabarovsk district, Russia; and 7-18, Amur region, Russia. A DNeasy plant mini kit (Qiagen, Germany) for genomic DNA extraction, according to the manufacturer's manual, was used. Quality and

quantity of the extracted DNA was checked with a UV/VIS spectrophotometer (Genesys 10S, Thermo Scientific, USA). The A_{260}/A_{280} of extracted DNA ranged from 1.7-2.0.

Isoinhibitor-specific oligonucleotide primers used for PCR amplification of BBI-A, BBI-C, and BBI-D are reported in Table 1, as well as the oligonucleotide primers for KTI3 gene. Endogenous gene lectin was used as quality control for DNA and PCR efficiency.

PCR was carried out using premix of 2x PCR Master Mix (Fermentas, Vilnius, Lithuania), with final concentration of 2 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} dNTP, and 1.25 units Taq DNA Polymerase (recombinant). PCR was performed in a final volume of 25 µL with 0.2 pmol μL^{-1} primers and approxmately 50 ng DNA. Amplifications were carried out in a Mastercycler ep gradient S thermal cycler (Eppendorf, Hamburg, Germany) under the following touchdown program: initial denaturation at 94°C for 3 min, followed by a touchdown program for 7 cycles, with successive annealing temperature decrements of 1°C in every cycle. For these first 7 cycles, the reaction was denatured at 94°C for 50 s, followed by annealing at 62°C → 56°C for 50 s, and polymerization at 72°C for 1 min and 30 s. The 30 subsequent cycles for amplification were similar, except for annealing temperature, which was 56°C for 50 s.

Amplified fragments for BBI-A were further subjected to digestion by *HindIII* restriction enzyme (Fermentas, Vilnius, Lithuania) (Deshimaru et al., 2004), while amplified fragments for KTI3 were digested with *Mse* I (*Tru1* I) restriction enzyme (Fermentas, Vilnius, Lithuania) (Wang et al., 2008). Amplification and restriction fragments were determined using electrophoresis on 2% agarose gel containing ethidium bromide (0.5 g mL^{-1}). The expected size of the amplified fragments was estimated by comparison with O'RangeRuler 50 bp DNA Ladder and FastRuler DNA Ladder, Low Range (Fermentas, Vilnius, Lithuania).

Table 1. Oligonucleotide primers used for PCR amplification of lectin, KTI3, BBI-A, BBI-C, and BBI-D genes.

Gene	Sequence (5'→3')	Amplicon (bp)	Reference
Lectin	GACGCTATTGTGACCTCCTC GAAAGTGTCAAGCTTAACAGCGACG	318	Meyer et al. (1996)
KTI3	AGTCCCGATTCTCCCAACA AGTACTCTCACACTTGTGTC	700	Jofuku et al. (1989)
BBI-A	ACATGGTGGTGCTAAAGGTGTGTT CTTGTTCATTAGTAGTTTTCCTTGTCA	350	Wang et al. (2008)
BBI-C	GACACTTGACAGGAAAAACAG GCCAAAAGCAAATTACTGGCC	480	Wang et al. (2008)
BBI-D	ACAGCAAAAACAACTAATAAAG TAAAAATGACCAAATTTGCT	550	Wang et al. (2008)

The agarose gel was visualized using UV transilluminator, and the images were captured with DOC II PRINT system (Vilber Lourmat, Marne-la-Valleé, France).

Plant juice extracts with CEL I activity were prepared as described by Till et al. (2006), for purification of CEL I. Only the extraction, salting out, and dialysis steps of the purification protocol were performed. Store-bought celery stalks (about 0.7 kg) were juiced at 4°C. Celery juice was adjusted to reach a final concentration of 0.1 mol L^{-1} Tris-HCl, pH 7.7, 100 µmol L^{-1} PMSF and 0.01% Triton X-100. The obtained solution was then centrifuged for 20 min at 10,000 *g* to pellet debris. Supernatant was brought to 25% saturation in $(NH_4)_2SO_4$, mixed for 1 hour at 4°C, and centrifuged at 10,000 *g*, at 4°C for 45 min. Resulting supernatant was adjusted to 80% with $(NH_4)_2SO_4$, mixed for 1 hour at 4°C, and centrifuged at 10,000 *g* for 1.5 hour. Pellet was suspended in buffer with 0.1 mol L^{-1} Tris-HCl pH 7.7, 100 µmol L^{-1} PMSF, 0.01% Triton X-100 (1/10 of starting plant juice extract volume). Suspension was dialyzed against the same buffer over night. Extract aliquots were stored at aproximately 20°C.

To form the heteroduplex, KTI PCR products from Kunitz variety – lacking active KTI – and from other soybean varieties were mixed in 1:1 ratio and subjected to heating and re-annealing process, running the following program: 95°C for 2 min; 95°C ramping to 85°C (-2°C per second); 85°C ramping to 25°C (-0.3°C per second); and 4°C hold, to form heteroduplex.

For CEL I digestion, the 10 µL of heteroduplexes were incubated in 5 µL of buffer D (20 mmol L^{-1} Tris–HCl, pH 7.4, 25 mmol L^{-1} KCl, 10 mmol L^{-1} $MgCl_2$) with 5 µL purified plant extract with CEL I (0.01 µg) at 45°C for 35 min (Oleykowski et al., 1998). The reaction was stopped with 5 µL of 0.15 mol L^{-1} EDTA. The digested products were determined using electrophoresis on 2% agarose gel, as previously described.

Results and Discussion

Soybean KTI has several polymorphic types, which are controlled by codominant multiple alleles at a single locus (Wang et al., 2008). Three of the KTI

genes (KTI1, KTI2, and KTI3) have been cloned and sequenced (Krishnan, 2001). A sequence for KTI was amplified by PCR (Figure 1) using a set of two primers designed on the basis of DNA sequences of KTI3 (= *Tia*) (Jofuku et al., 1989). A fragment of aproximately 700 bp was amplified in all analyzed varieties from the USDA germplasm collection, including the Kunitz variety – a genotype lacking active trypsin inhibitor.

The null phenotype of KTI is due to a mutation in the Kunitz trypsin inhibitor structural gene, and it is inherited as a recessive allele *ti*. It has reduced amounts of Kunitz trypsin inhibitor protein and lack detectable Kunitz trypsin inhibitor activity (Orf & Hymowitz, 1979). Therefore, one of the methods for detecting mutation and SNP is a heteroduplex mismatch cleavage assay using the endonuclease CEL I from celery (Zolala et al., 2009). The ability of celery juice extract CEL I to detect a mismatch at one or more nucleotide positions, without prior knowledge about this sequence, was shown by Oleykowski et al. (1998). The laboratory has purified this enzyme according to Till et al. (2006), which made the mutation detection assay less expensive. The enzyme is found to be extremely stable during purification, storage, and assay. Upon digestion of formed heteroduplexes with CEL I enzyme, the digested products were visualized in 2% agarose gels, avoiding the need for labeled primers, polyacrylamide gels, and DNA sequencers used in earlier versions of the methods (Galeano et al., 2009). The method for detecting SNPs in stress-related genes in rice, using CEL I and agarose gels, provide results which perfectly corresponded to the ones from polyacrylamide- and LI-COR-based analyses (Raghavan et al., 2007). Chen et al. (2011) also showed that agarose gels could be convenient for detecting SNP in common wheat. Moreover, the PCR reaction is cheaper because the primers used are not labelled.

Digestion of the heteroduplexes formed for KTI gene with CEL I enzyme generated bands with 500 bp and approximately 250 bp, in addition to full-lenght uncleaved product of 700 bp (Figure 2). The sum of the cleaved fragments is theoretically about the length of the PCR product (Oleykowski et al., 1998). Two obtained fragments (Figure 2) indicate the presence of SNP in Kunitz variety KTI gene, confirming that assay with the CEL I crude extract isolated in laboratory has the potential to identify SNP mismatches.

Restriction enzyme *Mse* I (*Tru1* I) was used to determine which KTI type was present. *Tia* type alleles had two restriction sites resulting in three fragments. *Tib* type had one restriction site resulting in two fragments (Wang et al., 2008). *Tib* type was found in cultivated and in wild soybean varieties (Figure 3). Electrophoretic forms *Tia*, *Tib*, *Tic*, and *Tid* have been reported in cultivated soybeans (Hymowitz, 1973;

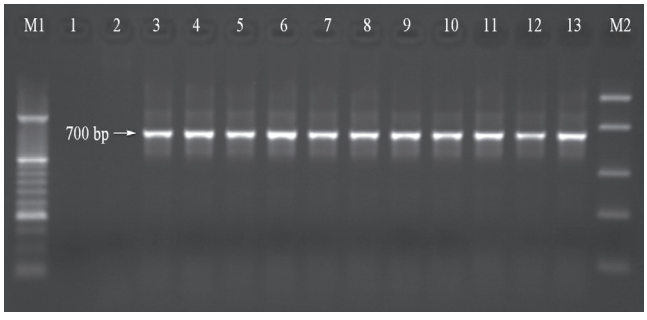

Figure 1. Agarose gel electrophoresis of PCR products for KTI. M_1, O'RangeRuler, 50 bp DNA Ladder, 50-1000 bp; 1. blank, no template control; 2. negative control, maize; 3. positive control, 'Vojvodjanka'; 4. 'Altona'; 5. 'Wilkin'; 6. 'Amsoy 71'; 7. 'Panther'; 8. 'Kunitz'; 9. 'Vojvodjanka'; 10. 'Fortuna'; 11. *Glycine soja* 37-2; 12. *Glycine soja* 42-2; 13. *Glycine soja* 7-18; M^2, Low Range DNA Ladder, 50-1500 bp.

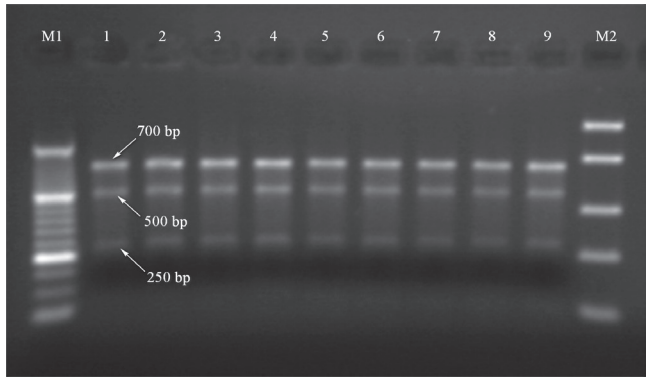

Figure 2. Agarose gel electrophoresis of heteroduplex mismatch cleavage CEL I endonuclease products in KTI gene. Mutants could be identified as those products that showed cleaved bands (500 bp and 250 bp), in addition to the full-length, uncleaved product (700 bp). M_1, O'RangeRuler, 50 bp DNA Ladder, 50-1000 bp; 1. 'Kunitz' vs. 'Altona'; 2. 'Kunitz' vs. 'Wilkin'; 3. 'Kunitz' vs. 'Amsoy' 71; 4. 'Kunitz' vs. 'Panther'; 5. 'Kunitz' vs. 'Vojvodjanka'; 6. 'Kunitz' vs. 'Fortuna'; 7. 'Kunitz' vs. *Glycine soja* 37-2; 8. 'Kunitz' vs. *Glycine soja* 42-2; 9. 'Kunitz' vs. *Glycine soja* 7-18; M^2, Low Range DNA Ladder, 50-1500 bp.

Zhao & Wang, 1992). Hymowitz (1973) determined that 89% of USDA soybean collection contained the *Tia* variant. In contrast, *Tic* was found only in 0.3% of the collection, and it commonly exists in cultivated soybeans (Wang et al., 2008). Wang et al. (2010) suggests that both wild and cultivated soybean usually contain the most commonly occurring *Tia* and *Tib* types, while the *Tid* form was found in just one Chinese cultivar. Lee et al. (2012) also found *Tia* and *Tib* as the predominant types. Unlike the digestion with CEL I, amplification of KTI products, followed by digestion with restriction enzymes, was not able to detect differences between *ti*-null and other types of *Ti* alleles.

Based on their structural features and inhibitory characteristics, BBIs are grouped in three main types – BBI-A, BBI-C, and BBI-D – and consist in a multigene family (Deshimaru et al., 2004). Using cultivated soybean and wild soybean genomic DNA as templates, fragments of 350 bp for BBI-A, 480 bp for BBI-C, and 550 bp for BBI-D were amplified (Figure 4). There were no variations in the amplificon sizes among cultivated and wild soybean samples, for all three inhibitors. Based on sequence comparisons, Wang et al. (2008) suggests that both wild and cultivated soybean had similar BBI genes. This is probably due to the close phylogenetic relation between the two species (Deshimaru et al., 2004).

BBI-A was further divided into two subtypes – A1 and A2 – according to small differences in their nucleotide sequences (Deshimaru et al., 2004). Amplified fragments for BBI-A were further subjected to digestion by *HindIII* restriction enzyme (Figure 5), for which only the coding sequence for BBI-A1 contains a clevage site, resulting in two fragments (Deshimaru et al., 2004). Between the USA-origin soybean, only 'Amsoy 71' showed the presence of

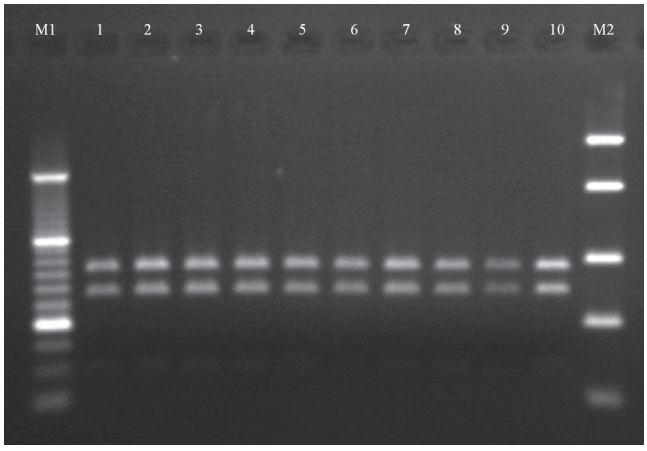

Figure 3. PCR-RFLP profiles by *Mse* I (*Tru1* I) restriction digest of the KTI gene. *Tib* type has one restriction site. M_1, O'RangeRuler, 50 bp DNA Ladder, 50-1000 bp; 1. 'Altona'; 2. 'Wilkin'; 3. 'Amsoy' 71; 4. 'Panther'; 5. 'Kunitz'; 6. 'Vojvodjanka'; 7. 'Fortuna'; 8. *Glycine soja* 37-2; 9. *Glycine soja* 42-2; 10. *Glycine soja* 7-18; M^2, Low Range DNA Ladder, 50-1500 bp.

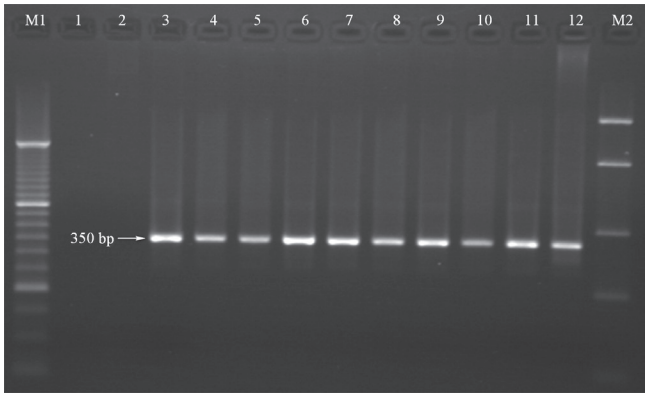

Figure 4. Agarose gel electrophoresis of PCR products for BBI-A. M_1, O'RangeRuler, 50 bp DNA Ladder, 50-1000 bp; 1. blank, no template control; 2. negative control, maize; 3. 'Altona'; 4. 'Wilkin'; 5. 'Amsoy 71'; 6. 'Panther'; 7. 'Kunitz'; 8. 'Vojvodjanka'; 9. 'Fortuna'; 10. *Glycine soja* 37-2; 11. *Glycine soja* 42-2; 12. *Glycine soja* 7-18; M^2, Low Range DNA Ladder, 50-1500 bp.

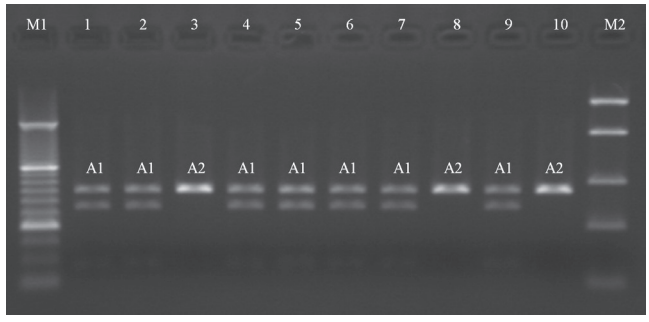

Figure 5. PCR-RFLP profiles by *HindIII* restriction digest of the BBI-A gene. BBI-A1 subtype contains a clevage site. M_1, O'RangeRuler, 50 bp DNA Ladder, 50-1000 bp; 1. 'Altona'; 2. 'Wilkin'; 3. 'Amsoy 71'; 4. 'Panther'; 5. 'Kunitz'; 6. 'Vojvodjanka'; 9. 'Fortuna'; 10. *Glycine soja* 37-2; 11. *Glycine soja* 42-2; 12. *Glycine soja* 7-18; M^2, Low Range DNA Ladder, 50-1500 bp.

A2 subtype, while two varieties from the Institute of Field and Vegetable Crops had this subtype. Out of the three wild soybean genotypes, just one had A1 subtype (Figure 5). Deshimaru et al. (2004) suggests that these two subtypes for BBI-A occur from distinct genes in the wild soybean genome, and not from polymorphic alleles in the genome.

Conclusions

1. There is a low level of genetic variation in 21-kD protein (KTI) and Bowman-Birk trypsin–chymotrypsin inhibitor (BBI) between the investigated varieties of cultivated and wild soybean.

2. The digestion of KTI products with restriction enzymes show that *Tib* type of KTI is a dominant type among the analyzed varieties, but it is not able to detect differences between *ti*-null and other types of *Ti* alleles.

3. The digestion method with celery extracts (CEL I) described here provide a simple and useful genetic tool for single nucleotide polymorphism (SNP) analysis.

Acknowledgments

To the Ministry of Education, Science and Technological Development of the Republic of Serbia, for support, by the projects TR-31024 and TR-31022.

References

BARROS, B. de A.; SILVA, W.G. da; MOREIRA, M.A.; BARROS, E.G. de. In silico characterization and expression analysis of the multigene family encoding the Bowman-Birk protease inhibitor in soybean. **Molecular Biology Reports**, v.39, p.327-334, 2012. DOI: 10.1007/s11033-011-0742-1.

BARROS, J.G. de A.; MORAES, R.M.A. de; PIOVESAN, N.D.; BARROS, E.G. de; MOREIRA, M.A. Efeito do inibidor de protease Kunitz sobre níveis de lipoxigenases em sementes de soja. **Ciência e Agrotecnologia**, v.32, p.1126-1132, 2008. DOI: 10.1590/S1413-70542008000400014.

CHEN, L.; WANG, S.-Q.; HU, Y.-G. Detection of SNPs in the VRN-A1 gene of common wheat (*Triticum aestivum* L.) by a modified Ecotilling method using agarose gel electrophoresis. **Australian Journal of Crop Science**, v.5, p.318-326, 2011.

CRUZ, A.C.B.; MASSENA, F.S.; MIGLIOLO, L.; MACEDO, L.L.P.; MONTEIRO, N.K.V.; OLIVEIRA, A.S.; MACEDO, F.P.; UCHOA, A.F.; GROSSI DE SÁ, M.F.; VASCONCELOS, I.M.; MURAD, A.M.; FRANCO, O.L.; SANTOS, E.A. Bioinsecticidal activity of a novel Kunitz trypsin inhibitor from Catanduva (*Piptadenia moniliformis*) seeds. **Plant Physiology and Biochemistry**, v.70, p.61-68, 2013. DOI: 10.1016/j.plaphy.2013.04.023.

DESHIMARU, M.; YOSHIMI, S.; SHIOI, S.; TERADA, S. Multigene family for Bowman-Birk type proteinase inhibitors of wild soja and soybean: the presence of two BBI-A genes and pseudogenes. **Bioscience, Biotechnology, and Biochemistry**, v.68, p.1279-1286, 2004. DOI: 10.1271/bbb.68.1279.

FUSARI, C.M.; LIA, V.V.; NISHINAKAMASU, V.; ZUBRZYCKI, J.E.; PUEBLA, A.F.; MALIGNE, A.E.; HOPP, H.E.; HEINZ, R.A.; PANIEGO, N.B. Single nucleotide polymorphism genotyping by heteroduplex analysis in sunflower (*Helianthus annuus* L.). **Molecular Breeding**, v.28, p.73-89, 2011. DOI: 10.1007/s11032-010-9462-9.

GALEANO, C.H.; GOMEZ, M,; RODRIGUEZ, L.M.; BLAIR, M.W. CEL I nuclease digestion for SNP discovery and marker development in common bean (*Phaseolus vulgaris* L.). **Crop Science**, v.49, p.381-394, 2009. DOI: 10.2135/cropsci2008.07.0413.

HYMOWITZ, T. Electrophoretic analysis of SBTI-A2 in the USDA soybean germplasm collection. **Crop Science**, v.13, p.420-421, 1973. DOI: 10.2135/cropsci1973.0011183X001300040008x.

JOFUKU, K.D.; GOLDBERG, R.B. Kunitz trypsin inhibitor genes are differently expressed during soybean life cycle and in transformed tobacco plants. **The Plant Cell**, v.1, p.1079-1093, 1989. DOI: 10.1105/tpc.1.11.1079.

JOFUKU, K.D.; SCHIPPER, R.D.; GOLDBERG, R.B. A frameshift mutation prevents Kunitz trypsin inhibitor mRNA accumulation in soybean embryos. **The Plant Cell**, v.1, p.427-435, 1989. DOI: 10.2307/3869103.

KIM, D.S.; LEE, K.J.; KIM, J.-B.; KIM, S.H.; SONG, J.Y.; SEO, Y.W.; LEE, B.-M.; KANG S.-Y. Identification of Kunitz trypsin inhibitor mutations using SNAP markers in soybean mutant lines. **Theoretical and Applied Genetics**, v.121, p.751-760, 2010. DOI: 10.1007/s00122-010-1346-1.

KRISHNAN, H.B. Characterization of a soybean [*Glycine max* (L.) Merr.] mutant with reduced levels of Kunitz trypsin inhibitor. **Plant Science**, v.160, p.979-986, 2001. DOI: 10.1016/S0168-9452(01)00346-6.

KUROYANAGI, M.; KATAYAMA, T.; IMAI, T.; YAMAMOTO, Y.; CHISADA, S.;, YOSHIURA, Y.; USHIJIMA, T.; MATSUSHITA, F.; NOZAWA, A.; SUZUKI, Y.; KIKUCHI, K.; OKAMOTO, H. New approach for fish breeding by chemical mutagenesis: establishment of TILLING method in fugu (*Takifugu rubripes*) with ENU mutagenesis. **BMC Genomics**, v.14, n.786, 2013. DOI: 10.1186/1471-2164-14-786.

LEE, K.J.; KIM, J.-B.; HA, B.-K.; KIM, S.H.; KANG, S.-Y.; LEE, B.-M.; KIM, D.S. Proteomic characterization of Kunitz trypsin inhibitor variants, Tia and Tib, in soybean [*Glycine max* (L.) Merrill]. **Amino Acids**, v.43, p.379-388, 2012. DOI: 10.1007/s00726-011-1092-y.

LIVINGSTONE, D.; VADIM, B.; MARINA, K.; MONICA, A.S.; ELIO, M.H.; NIELS, C.N. Reduction of protease inhibitor activity by expression of a mutant Bowman-Birk gene in soybean seed. **Plant Molecular Biology**, v.64, p.397-408, 2007. DOI: 10.1007/s11103-007-9163-x.

MEYER, R.; CHARDONNENS, F.; HÜBNER, P.; LÜTHY, J. Polymerase chain reaction (PCR) in the quality and safety assurance of food: detection of soya in processed meat products.

Zeitschrift für Lebensmittel-Untersuchung und-Forschung A, v.203, p.339-344, 1996. DOI: 10.1007/BF01231072.

OLEYKOWSKI, C.A.; MULLIS, C.R.B.; GODWIN, A.K.; YEUNG, A.T. Mutation detection using a novel plant endonuclease. **Nucleic Acids Research**, v.26, p.4597-4602, 1998. DOI: 10.1093/nar/26.20.4597.

ORF, J.H.; HYMOWITZ, T. Inheritance of the absence of the Kunitz trypsin inhibitor in seed protein of soybeans. **Crop Science**, v.19, p.107-109, 1979. DOI: 10.2135/cropsci1979.0011183X001900010026x.

RAGHAVAN, C.; NAREDO, M.E.B.; WANG, H.; ATIENZA, G.; LIU, B.; QIU, F.; McNALLY, K.L.; LEUNG, H. Rapid method for detecting SNPs on agarose gels and its application in candidate gene mapping. **Molecular Breeding**, v.19, p.87-101, 2007. DOI: 10.1007/s11032-006-9046-x.

RAKASHANDA, S.; AMIN, S. Proteases as targets in anticancer therapy using their inhibitors. **Journal of Life Sciences**, v.5, p.133-138, 2013.

SONG, J.Y.; PIAO, X.-M.; CHOI, Y.-M.; LEE, G.-A.; CHUNG, J.-W.; LEE, J.-R.; JUNG, Y.; PARK, H.-J.; LEE, M.C. Evaluation of genetic diversity and comparison of biochemical traits of soybean (*Glycine max* L.) germplasm collections. **Plant Breeding and Biotechnology**, v.1, p.374-384, 2013. DOI: 10.9787/PBB.2013.1.4.374.

TILL, B.J.; ZERR, T.; BOWERS, E.; GREENE, E.A.; COMAI, L.; HENIKOFF, S. High-throughput discovery of rare human nucleotide polymorphisms by Ecotilling. **Nucleic Acids Research**, v.34, p.e99, 2006. DOI: 10.1093/nar/gkl479.

WANG, K.J.; LI, X.H.; TAKAHATA, Y.; YAMASHITA, T. Allelic diversity and geographical pattern at soybean Kunitz trypsin inhibitor single locus in Chinese wild soybean (*Glycine soja* Sieb. & Zucc.). **Plant Breeding**, v.129, p.264-270, 2010. DOI: 10.1111/j.1439-0523.2009.01734.x.

WANG, K.J.; TAKAHAKA, Y.; KONO, Y,; KAIZUMA, N. Allelic differentiation of Kunitz trypsin inhibitor in wild soybean (*Glycine soja*). **Theoretical and Applied Genetics**, v.117, p.565-573, 2008. DOI: 10.1007/s00122-008-0800-9.

WANG, K.J.; YAMASHITA, T.; WATANABE, M.; TAKAHATA, Y. Genetic characterization of a novel Tib-derived variant of soybean Kunitz trypsin inhibitor detected in wild soybean (*Glycine soja*). **Genome**, v.47, p.9-14, 2004. DOI: 10.1139/g03-087.

WANG, Y.P.; CHEN, X.T.; QIU, L.J. Novel alleles among soybean Bowman-Birk proteinase inhibitor gene families. **Science in China Series C - Life Sciences**, v.51, p.687-692, 2008. DOI: 10.1007/s11427-008-0096-7.

YANG, W.; BAI, X.D.; KABELKA, E.; EATON, C.; KAMOUN, S.; VAN DER KNAAP, E.; DAVID, F. Discovery of single nucleotide polymorphisms in *Lycopersicon esculentum* by computer aided analysis of expressed sequence tags. **Molecular Breeding**, v.14, p.21-34, 2004. DOI: 10.1023/B:MOLB.0000037992.03731.a5.

ZHAO, S.W.; WANG, H. A new electrophoretic variant of SBTi-A2 in soybean seed protein. **Soybean Genetics Newsletter**, v.19, p.22-24, 1992.

ZOLALA, J.; BAHRAMI, A.-R.; FARSI, M.; MATIN, M.M.; YASSAEE, V.-R. Comparison of CEL I gene expression and mismatch-cleavage activity in some *Apiaceae* plants. **Molecular Breeding**, v.24, p.17-24, 2009. DOI: 10.1007/s11032-009-9267-x.

Received on October 2, 2013 and accepted on January 30, 2014